

STUDIES OF THE EFFECTS OF ETHIONINE, AND PHENOBARBITAL
ON THE PHOSPHATIDYLCHOLINES OF RAT LIVER

by

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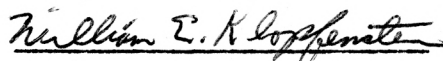
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INTRODUCTION

Phospholipids are an integral part of most mammalian membranes. Phosphatidylcholine has been shown to be a major component of liver microsomal membranes, and the enzymes responsible for its biosynthesis are located primarily in the endoplasmic reticulum. The distribution of molecular species appears to be related to the membrane function, regulating such things as transport and permeability. Unsaturation in the fatty acid chains increases the permeability of the membrane, the fluidity of which is usually controlled by the cholesterol content.

Various drugs are known to alter lipid metabolism. The effects of phenobarbital, a C-5 ethyl, phenyl derivative of barbituric acid, and ethionine, an ethyl analogue of methionine, on phosphatidylcholine biosynthesis have been studied. Phenobarbital seems to decrease the catabolism of phosphatidylcholine, with some reports of no changes in the percentages of the fatty acids present, and other reports that decreases occur in some of the more unsaturated fatty acids such as arachidonic and docosahexenoic. Ethionine has been shown to decrease the percentage of arachidonic and docosahexenoic and increase linoleic and oleic acids.

The isolation of phosphatidylcholine from the livers of drug treated animals, and the separation of the trimethylsilyl

diglyceride derivatives by gas chromatography can give an indication of the changes these drugs bring about in the synthesis of the molecular species of phosphatidylcholine.

STATEMENT OF THE PROBLEM

Phenobarbital and ethionine are known to produce changes in phosphatidylcholine biosynthesis. Phosphatidylcholine is a major constituent of most mammalian membranes, and as the structure and function of the membrane is a product of the composition of the membrane, it is important to know what effect these drugs have on the composition of phosphatidylcholine. This research is an attempt to determine the modifications in molecular species of phosphatidylcholine brought about by these drugs. Gas chromatography of the trimethylsilyl derivatives of the diglycerides obtained from liver phosphatidylcholines of the treated animals will be used as the analytical tool. From the shifts in percentages of molecular species present, speculation about how the drugs are acting on the biosynthetic enzymes will be made.

LITERATURE REVIEW

The discovery of phospholipids as a major constituent of many animal membranes has led to extensive investigations of their exact composition under both normal and abnormal conditions. The possibility that abnormal conditions alter the phospholipid composition, which in turn alters the membrane properties, has intrigued many scientists. The understanding of the normal composition and the pathways of phospholipid metabolism is necessary before comparison to abnormal conditions can be made.

Phosphatidylcholine has been shown to be a major constituent of rat liver membranes (1,2), and is synthesized mainly by two pathways. The major one, known as the Kennedy pathway (3), involves the transfer of the phosphorylcholine moiety from cytidine diphosphate choline (CDP-choline) to a 1,2-diacyl-sn-glycerol, forming phosphatidylcholine and cytidine monophosphate (CMP). The enzyme catalyzing this reaction is CDP-choline:1,2 diglyceride cholinephosphotransferase (EC.2.7.8.2). This pathway accounts for some 80-90% of the phosphatidylcholine synthesized by the rat liver microsomes (4). The minor pathway, the Bremer-Greenberg pathway (5), involves the sequential methylation of phosphatidylethanolamine by the transfer of methyl groups from S-adenosyl-L-methionine to produce phosphatidylcholine and S-adenosylhomocysteine. There

are two enzymes involved. One catalyzes the addition of the first methyl group, and the other catalyzes the last two additions. The enzymes are referred to as S-adenosyl-L-methionine: phosphatidylethanolamine methyltransferases (SAME:PE methyltransferase). All three enzymes are confined to the endoplasmic reticulum (6,7).

In an extensive study by Hill et al. (8), the diacyl subspecies of phosphatidylcholine and phosphatidylethanolamine in rat liver were identified. In 1,2-diacyl-sn-glycero-3-phosphorylcholine the monoene, diene, tetraene and hexaene species represented 11, 28, 35, and 5 mole percent respectively, whereas in 1,2-diacyl-sn-glycero-3-phosphoryl-ethanolamine the corresponding values were 2, 19, 48, and 19 mole percent. Furthermore, the tetraene subspecies of 3-sn-phosphatidylethanolamine which contained arachidonic acid showed a 2:1 preference for stearic over palmitic as the saturated acid in position 1. In the hexaene subspecies, the 1:2 ratio of stearic to palmitic indicated a preference for palmitic acid in position 1. In both monoene and diene subspecies a preference for palmitic over stearic was seen.

Lyman et al. (9) demonstrated that the fastest moving subfraction of phosphatidylcholine from whole rat liver on thin layer plates resembled the fraction of phosphatidylethanolamine. This subfraction had a higher percentage of stearic and arachidonic acid than the slower subfractions. Females had a higher percentage of this subfraction than did males.

These authors suggested that a large percentage of the stearic-arachidonic phosphatidylethanolamine was methylated to produce phosphatidylcholine, and that there was a higher rate of methylation in females. Lyman et al. (10) were able to show that estradiol was the agent that enhances the methylation pathway. Studies by Rytter et al. (11) demonstrated that ^{14}C -choline was incorporated mainly into phosphatidylcholine with two or less double bonds, and ^{14}C -ethanolamine was mainly incorporated into phosphatidylcholine with six double bonds. Arvidson (12) found that the tetraene subfraction is dominant in both phosphatidylethanolamine and phosphatidylcholine, and that the hexaene fraction was more prominent in phosphatidylethanolamine than phosphatidylcholine. He reported that ^{14}C -choline was incorporated most rapidly into 16:0, 18:1 and 16:0, 18:2 diglycerides, but slowly into 18:0, 20:4 diglycerides. On the other hand, ^{14}C -methionine was incorporated most rapidly into the tetra- and hexaenoic fractions. Tinoco et al. (13) supported this work by showing that the subfractions with 18:2 and 20:4 in the 2 position had 88 percent of the total radioactivity in phosphatidylcholine after ^{14}C -methionine injection into female rats. Kanoh (14), using this same labelled isotope, confirmed that hexaenoic phosphatidylcholine was the major product of the methylation of phosphatidylethanolamine.

Though the Kennedy pathway shows a preference for the diglycerides with two or less double bonds, Mudd et al. (15)

found that the enzyme exhibited no specificity for any particular diglycerides. Van Golde et al. (16) were able to show that de novo synthesis produced mainly mono- and diunsaturated phosphatidylcholines, and that arachidonic acid was introduced by transacylation of lysophosphatidylcholine. Trehella and Collins (17) confirmed that the 18:0, 20:4 diglyceride was formed largely via the methylation of phosphatidylethanolamine to phosphatidylcholine, and that the 16:0, 18:2 diglyceride was formed mainly by the CDP-choline pathway.

Choline deficiency has been shown to decrease the 20:4 fraction of phosphatidylcholine, and increase the 18:1, 18:2 and 22:6 fractions (18). Other workers (19,20) have also indicated a decrease in 20:4 in both phosphatidylcholine and phosphatidylethanolamine when rats are fed a choline deficient diet. Beare-Rogers (19) reported that the percentage increases in 22:5 and 22:6 served as replacements for the 20:4 in phosphatidylethanolamine, but did not become major constituents of phosphatidylcholine. She also reported that the relative distribution of 20:4 between phosphatidylethanolamine and phosphatidylcholine in choline deficient animals remained the same as in control animals, but less phosphatidylcholine was synthesized and less phosphatidylethanolamine contained 20:4. Lyman et al. (20) supported these observations by reporting that choline deficiency reduced the proportion of 20:4 in the phosphatidylethanolamines of animals of both sexes, and increased the proportion of 22:5.

Turkki and Silvestre (21) demonstrated that choline deficiency stimulated the methylation of phosphatidylethanolamine to phosphatidylcholine as measured by ^{14}C -methionine incorporation. Glenn and Austin (22) showed that choline deficiency stimulated an increase in methylation of phosphatidylethanolamine to phosphatidylcholine in both males and females, with males showing an increase in the activity of SAME:PE methyltransferase after only 21 hours. Females showed an increase in activity of this enzyme only after three to six weeks. Recently Skurdal and Cornatzer (23) found that choline deficiency produced a decrease in the activity of this enzyme in females, but did not alter the choline phosphotransferase.

In accordance with Lyman's findings that estradiol was the causative agent for the increased methylation in females, Young (24) has demonstrated that estradiol enhanced the activity of SAME:PE methyltransferase in castrated male rats and decreased the CDP-choline:1,2 diglyceride cholinephosphotransferase activity.

Just recently (25), it has been shown that the back reaction of the Kennedy pathway is important in the degradation of phosphatidylcholine to form 1,2-diacyl-sn-glycerols which are more unsaturated than those formed from phosphatidic acid. The ethanolamine phosphotransferase utilized mainly hexaenoic diacylglycerols to form phosphatidylethanolamine, and this species was also the main species to be

methyated to form phosphatidylcholine. This may explain the observation by Arvidson (12) that the disappearance of ^{14}C -methionine label occurred most rapidly in the hexaenoic subfraction of phosphatidylcholine.

It is apparent that both pathways are influenced by the availability of substrates, donors, and the activities of the enzymes, and that these factors can modify the diglyceride species of phosphatidylcholine produced.

The well-known effects of ethionine are an indication of the flexibility of these pathways. Simmonds et al. (26) showed that ethionine inhibited SAME:PE methyltransferase, and Stekol et al. (27) found that ethionine inhibited methionine methyl incorporation into phosphatidylethanolamine to form phosphatidylcholine. Ulsamer and Glenn (28), and Sato and Hasegawa (29) reported that ethionine increased the ^{32}P uptake into phosphatidylcholine and brought about a drop in phosphatidylethanolamine labelling in females. Lyman et al. (30) showed that the diglycerides were in fact changed in males, and reported that the percentage of arachidonic acid was decreased and the oleic and linoleic acid percentages were increased. These results are consistent with what one would expect if the methylation of the highly unsaturated phosphatidylethanolamines were inhibited. Ethionine also impaired the synthesis of arachidonic acid from linoleic acid in the desaturation reaction, thus lowering the amount of highly unsaturated fatty acid available for phospholipid

synthesis.

The effect of phenobarbital on the pathways is not as well established as that of ethionine. Phenobarbital has many actions on the liver. It is a well-known inducer of many of the drug metabolizing enzymes such as dopa decarboxylase (31) and aminopyrine demethylase (32). Orrenius and Ericsson demonstrated that phenobarbital decreased the activity of glucose-6-phosphatase, and enhanced the formation of the endoplasmic reticulum (33). Phenobarbital has been shown to increase liver weight (32,34,35), increase total protein (32,36), and induce the formation of cytochrome P-450 (37,38). Cytochrome P-450 appears to be involved in the hydroxylating system of the microsomes, and is the binding site of the drug undergoing the reaction (37). Phosphatidylcholine is essential for the enzymatic reduction of cytochrome P-450, though it does not change the spectral properties or alter the binding of the substrates (39). The dramatic increase in the endoplasmic reticulum requires the synthesis of phospholipid for additional membrane. Just exactly how this phospholipid increase is brought about has been the subject of much controversy.

Using an orthophosphate (^{32}P) labelling technique, Orrenius et al. (40) reported that an increase in phospholipid synthesis in the liver microsomes was stimulated by phenobarbital. Holtzman and Gillette (41), using the same technique, found that the synthesis was not increased, but

that the accumulation was due to a decreased catabolism. This technique did not measure the methylation pathway or the conversion of lysophospholipids to phosphatidylcholine. Infante et al. (42) using both ^{32}P -phosphate and ^{14}C -palmitate were able to show that neither the Kennedy pathway nor the conversion of lysophospholipids to phosphatidylcholine was increased by phenobarbital. Though the methylation pathway was not directly measurable in their study, they did report that, since they recovered more label in the phosphatidylethanolamine of phenobarbital treated animals than in controls, it appeared that the conversion of phosphatidylethanolamine to phosphatidylcholine may have been decreased by phenobarbital. Their conclusion supported that of Holtzman in that a decreased catabolism appeared to be responsible for the phospholipid accumulation after phenobarbital treatment. Stein and Stein (43) were finally able to report that phenobarbital decreased the activity of acyl phosphohydrolase toward both phosphatidylethanolamine and phosphatidylcholine. They also reported that the conversion of phosphatidylethanolamine to phosphatidylcholine appeared to be decreased, and that there was no increase in incorporation of choline or ethanolamine. Recently, Ericksson and Dallner (44), also reported that phenobarbital produced a decreased breakdown of phosphatidylcholine as measured by the half life of ^3H -glycerol labelled phospholipid.

Young et al. (45) have reported that in vitro phenobar-

bital increased the activity of SAME:PE methyltransferase, but not that of choline phosphotransferase. They also reported an increased incorporation of ^3H -methyl from methionine into phosphatidylcholine. However, Davison and Wills (46) have demonstrated that phenobarbital gave an increase in SAME:PE methyltransferase within 12 hours, but brought about a decrease after three days. Thus, it is still not well established whether phenobarbital exerts its effects by only a decreased catabolism, or a combination of this and an increased synthesis. No reports of diglyceride species from phenobarbital treated animals have been published, though reports of fatty acid composition of phospholipids have been. Ariyoshi et al. (47) reported that no change in phosphatidylcholine fatty acids was brought about, though a decrease in stearic acid of phosphatidylethanolamine was noted. Davison and Wills (48) found that linoleic acid was increased, and arachidonic and docosahexenoic acids were decreased. If phenobarbital does enhance the methylation pathway, the diglycerides formed should be different than the controls. Since ethionine inhibits this pathway, the differences between the ethionine treated and the phenobarbital treated animals might be considerable. Treatment of animals with both drugs could produce a cancellation effect, or one treatment might predominate over the other. If phenobarbital does not enhance the methylation pathway, but only decreases catabolism, the diglycerides accumulated might reflect the speci-

ficities of the catabolic enzymes.

The analysis of phospholipids has progressed dramatically over the last few years. Since the discovery and use of the phospholipases, much information has been collected. Treatment of the phospholipids with phospholipase A₂, known as phosphatide acyl-hydrolase (EC.3.1.1.4), releases the fatty acid at position 2 of the phospholipids whether or not the fatty acid is unsaturated (49). Some specificity for different phospholipids is present depending upon the source of the enzyme (50). Phospholipase A₁ removes the fatty acid esterified at position 1 of the phospholipids (51), while phospholipase B removes the remaining fatty acid from position 1 or 2 of lysolecithin. It tends to remove unsaturated fatty acids faster than saturated ones (52). Using these enzymes the fatty acids at positions 1 and 2 can be determined once the phosphatidylcholine has been purified. Using total phosphatidylcholine as the substrate one can measure the percentage of each fatty acid present at each position. However, this procedure does not tell which fatty acids occur together in one molecule. With the advent of argentation chromatography whereby the purified phosphatidylcholine is chromatographed on silica gel treated with silver nitrate (53), subfractions can be isolated which differ in the degree of unsaturation of their fatty acids. These subfractions can then be treated with the phospholipases to distinguish which fatty acids predominate in each subfraction. The phospho-

tidylcholine can first be treated with phospholipase C to remove the phosphorylcholine moiety (54), and then the diglycerides produced subjected to the argentation chromatography. However, the subfractions produced in either case are still heterogeneous, and one still has not been able to separate pure molecular species. The development of analysis of diglycerides by gas liquid chromatography (GLC) has permitted the analysis of molecular species without degradation of the diglyceride. It also has the advantage of eliminating one chromatographic step with the possibility of loss of minor components, and has reduced the time involved. O'Brien and Klopfenstein (55) were able to separate diglycerides on 3% OV-1, using a glass column with a special Kovar-to-glass seal. This system was able to separate 1,2 from 1,3 diglycerides and distinguish between unsaturation of at least two subfractions with a total of 36 carbons. Just recently Myher and Kuksis (56), using 3% Apolar-5CP, separated diglycerides differing in carbon number and in unsaturation. However, their system did not give baseline resolution of the higher carbon species that differed from each other in only one double bond. In addition, this system required an hour and a half for each run. Further progress in this area should yield faster, higher resolution systems, which will become significant tools in the analysis of molecular species of phosphoglycerides.

EXPERIMENTAL METHODS

Animal Treatments

Male Sprague Dawley rats obtained from our own colony, with a weight range of 217-247g for the first experiment and 253-408g for the second experiment were used. The animals were weighed and grouped prior to the beginning of the injections and labelled by notching either the right, left, or neither ear. The animals were arranged so that all groups had relatively equal weight distribution, with six rats per group and five rats per group in the first and second experiments, respectively. They were housed three animals to a large cage, kept in a room maintained at constant temperature (72° F) with 12 hours of alternating light and dark. They were fed ad libitum with Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.), and water was available at all times. One group of animals was injected intraperitoneally with 80 mg phenobarbital/kg of body weight once a day for three days, one group with 1 mg DL-ethionine/g of body weight divided into four separate doses given two hours apart on the third day, and one group with both drugs. Controls in both experiments received 0.9% saline containing the same concentration of propylene glycol as the phenobarbital solution. An additional control group in the second experiment received

0.9% saline with no propylene glycol added. The volume of saline received by the controls was proportional to the weight of the animal. The ethionine treated animals received 0.9% saline injections on the first two days of the experiment of a volume equal to the controls. A fresh needle (Monoject 200, 25 gauge by 5/8 inch, Sherwood Medical Industries, Inc., Deland, Florida) was used for each individual injection. Food was removed from all animals at the beginning of the ethionine injections. The animals were weighed again and then killed by decapitation on the morning of the fourth day. The livers were quickly removed, placed in pre-weighed plastic bags, weighed and placed on ice. They were frozen at -16° C until ready for extraction.

Preparation of Drug Solutions

The phenobarbital solutions were prepared by diluting a purchased stock solution of 162 mg phenobarbital/ml of a propylene glycol solution (Gotham Pharmaceutical Co., Inc., Brooklyn, N.Y.). Dilutions were made with a sterilized 0.9% saline solution to produce a concentration that would deliver the required dosage of 80 mg/kg in less than one milliliter. By assuming the vehicle to be pure propylene glycol, the final concentration of propylene glycol in the working solution could be calculated.

A 0.9% saline solution was made that contained the same

concentration of propylene glycol as the phenobarbital solution. The 0.9% saline solution was autoclaved at 15 psi, 248° C for 30 minutes.

The concentration of the ethionine (General Biochemicals, Inc., Chagrin Falls, Ohio) solution was limited by the low solubility of ethionine in water. Solutions of 25 mg ethionine/ml of 0.9% saline were prepared. In order to deliver the required dosage, volumes of up to four milliliters were injected.

Phosphatidylcholine Isolation

The liver lipids were extracted according to the method of Folch et al. (57). The livers were homogenized in a Waring blender at one-half speed for one to two minutes. The filtrate was removed by suction filtration. This was rotary evaporated and the residue redissolved in chloroform:methanol (2:1). The extract was put into snap top vials, anhydrous Na_2SO_4 was added, the vials flushed with nitrogen and then stored at -16° C. Two livers were pooled for most samples in both experiments.

Attempts to chromatograph these extracts on one millimeter thick Silica Gel PF (Brinkman Instruments, Inc., Westbury, N.Y.) coated on glass plates 10 by 20 centimeters, in a solvent system of chloroform:methanol:water (65:25:4) resulted in overloading and inadequate separation of the lipids. In order to eliminate much of the neutral lipids, column

chromatography using Bio Sil BH (Calbiochem, Los Angeles, Calif.) made up in chloroform:methanol (2:1) was carried out. A column measuring 6.5 by 2.5 cm with a glass wool plug at the bottom was used. Sequential solvents used were 50 ml chloroform, 50 ml chloroform:methanol (4:1), 50 ml chloroform:methanol (1:1), 50 ml chloroform:methanol (1:4), and 50 ml methanol. This system gave fairly good separation with a flow rate of one to two milliliters per minute and collection of 10 ml fractions. Thin-layer chromatography of aliquots of each fraction established that the phosphatidylcholine was not eluted until fraction 10 or 11 and was completely eluted by fraction 20. Thus fractions 10-20 were subsequently collected from each sample. Chromatography in the chloroform:methanol:water (65:25:4) solvent system still did not give good separation of some of the phospholipids. A solvent system reported by Skipski et al. (58) was used in addition to using "basic" silica gel made by slurring the gel in 1 mM Na_2CO_3 . This system gave good separation of all the other phospholipid contaminants from phosphatidylcholine. The bands were revealed by inspection under ultraviolet light of wavelength 254 nanometers (Chromato-Vue, Model C-3, Ultraviolet Products, Inc., San Gabriel, Calif.). The bands were scraped and the phosphatidylcholine extracted from the gel with three washes of chloroform:methanol (2:1). The application of the sample to the plates, the chromatography, and the drying of the plates were all carried out in a nitrogen at-

mosphere to minimize oxidation of the lipids.

Preparation of Diglycerides

The extracts were evaporated to dryness under nitrogen and phospholipase C hydrolysis was carried out. The enzyme system employed 4-8 μ moles of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.0-4.0 mg phospholipase C (Clostridium welchii, Sigma Chemical Co., St. Louis, Mo.), 2-4 ml diethyl ether and 2-4 ml Tris buffer (pH 7.35, .02 M). It was important to dissolve the residue in the ether before adding the Tris buffer in order to obtain good hydrolysis. The reaction mixture was vigorously stirred in the dark at room temperature for 12-24 hours. A 12 hour reaction time produced less rearrangement of the 1,2 diglycerides to the 1,3 diglycerides than the 24 hour treatment. The enzyme reaction mixture was extracted with two washes of ether. The ether extracts were combined and evaporated to dryness and the residue redissolved in heptane. This solution was put under nitrogen and stored at -16°C .

Preparation of Diglyceride TMS Derivatives

The trimethylsilyl derivatives of the diglycerides obtained from the phosphatidylcholines were made by adding approximately .25 ml of N,O-Bis(trimethylsilyl)acetamide (Pierce Chemical Co., Rockford, Ill.) to .25-.50 ml of the

diglyceride solution in snap top vials. This mixture was allowed to react at room temperature for ten minutes, warmed slightly and concentrated under nitrogen.

Gas Chromatography of TMS Derivatives

Gas chromatography was carried out on a Barber Coleman 5000 gas chromatograph equipped with a hydrogen flame ionization detector, using an eight-foot by one-eighth inch column packed with 10% Apolar 10C (Applied Science Labs, State College, Pa.) on Chromosorb Q, 100/120 mesh. The column was operated isothermally at 265° C, with an injector temperature of 275° C, and a detector temperature of 300° C, using argon as the carrier gas with a flow rate of 40 ml/minute. An adequate volume (usually less than one μ l) of the derivative was used for each injection. Peak areas were determined by triangulation.

Gas Chromatography of Standards

Diglyceride standards were purchased and TMS derivatives made and chromatographed as previously described. X-glycerol-1,2-dimyristate, X-glycerol-1,2-dipalmitate, X-glycerol-1,2-diheptadecanoate, X-glycerol-1,2-distearate, X-glycerol-1,2-dinonadecanoate, X-glycerol-1,2-dipalmitoleate, X-glycerol-1,2-dilinoleate, and X-glycerol-1,2-dilinolenate

were purchased from Nu-Chek Prep., Inc., Elysian, Minn. X-glycerol-1,2-dioleate, sn-glycerol-2-stearate-3-oleate, and sn-glycerol-2-stearate-3-linoleate were purchased from Serdary Research Labs, London, Ontario, Canada. The TMS derivatives were chromatographed individually and then a pooled sample of all the standards was chromatographed.

DISCUSSION

The selection of the dosage and the duration of the treatment of the animals with phenobarbital was a compromise among the various treatments cited in the literature. Most treatments were from three to five days at dosages of 50-100 mg phenobarbital/kg of body weight (32,35,38,41,42,44,45,47,48). Argyris and Magmis have stated that the maximum effect of phenobarbital on liver weight peaks after three days of treatment, and the increase in demethylase activity is maximal after four days (32). The procedure employed was adequate to achieve the desired results, and was very similar to that of other workers so that comparisons of results can be made. The selection of the dosage and duration of ethionine treatment was made on a similar basis. Some workers (30) introduced the ethionine in the diet, whereas others (28,29,47,59) injected the ethionine in divided doses, to give a total dose of .75-1.0 mg ethionine/g of body weight.

In order to obtain an idea of the effect of the drugs on the liver weight, a liver wet weight to body weight ratio was calculated. This ratio is more meaningful than comparison of direct liver wet weight as there was a wide variation in body weight in some groups and between experiments which would also have an effect on the liver weight. The body weight used in these calculation was the weight obtained

prior to killing. As can be seen from Figure 1, all three drug treatments increased the ratio over that of the controls in both experiments. In addition, in each experiment a pattern of phenobarbital plus ethionine > ethionine > phenobarbital > controls is seen, though the increases in all cases in the second experiment were not as large as in the first experiment. This may be the result of a leveling off in the liver growth. The animals in the second experiment were litter mates of those in the first experiment, and thus were both older and heavier by the time the second experiment was carried out. It can also be seen that the phenobarbital plus ethionine treatment shows a slightly greater than additive effect. Columns I and K are the calculated ratios obtained by adding the increases over controls of phenobarbital and of ethionine. In both experiments these calculated ratios are less than the actual values, as depicted in columns H and J for the first and second experiments, respectively. This suggests that the drugs are acting at two different sites or by two different mechanisms to bring about the increase in liver weight. The identical control values shown by columns B and C in the second experiment indicate that the incorporation of propylene glycol has no effect over that of saline on the liver/body weight ratio. Thus, the propylene glycol in the phenobarbital solution does not exert any additional effect to that of the phenobarbital.

The procedures used in this research to isolate and

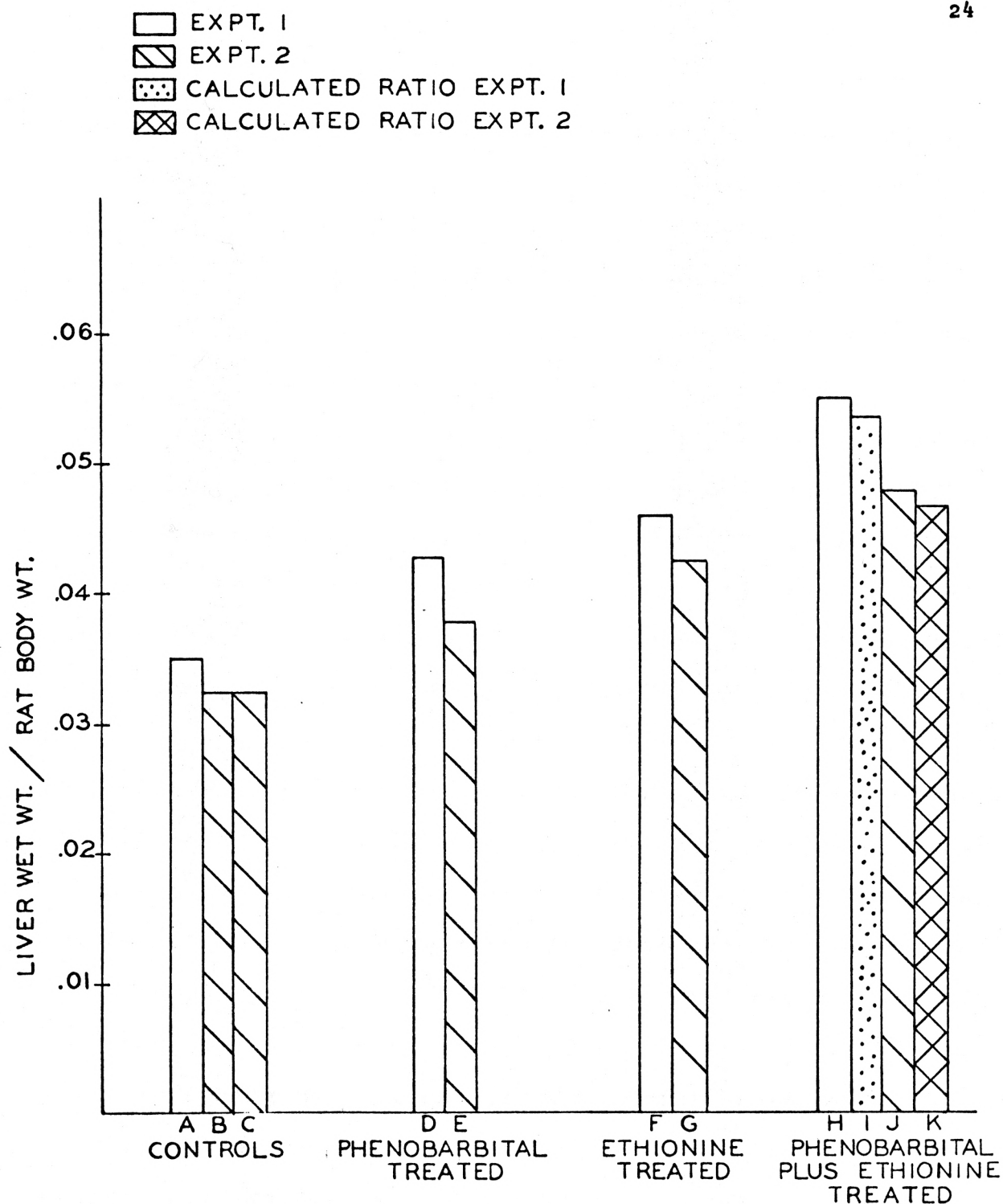


Figure 1. Liver wet weight to body weight ratios of treated animals.

prepare the diglycerides from the phosphatidylcholines of drug treated rats were very successful. The column chromatography of the total liver lipid extract gave good separation of the neutral lipids from the phospholipids. According to Ansell et al. (60), the separation of the phospholipids into classes by column chromatography has not yet been achieved, though some partial separation is possible. Thin layer chromatography, however, does give clean separation of the classes. There are many solvent systems available, though the one used by Skipski et al. (61) gives better separation of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, and lysophosphatidylcholine from phosphatidylcholine than other commonly used systems. The use of basic silica gel appears to play an important role in this separation. The base may act to "titrate" the more acidic phospholipids as they move up the plate, increasing the overall charge on the molecule, or the base could be titrating the acetic acid as it moves up the plate. Phosphatidylcholine does not have a group which is titratable under these conditions. The R_f values reported for this solvent system are .79 for phosphatidylethanolamine, .59 for phosphatidylserine, .48 for phosphatidylinositol, .32 for phosphatidylcholine, .19 for sphingomyelin, and .10 for lysophosphatidylcholine (60). Values calculated from sample plates fell within this range, with values generally running .75 for phosphatidylethanolamine, .62 for phosphatidylserine, .31 for phospho-

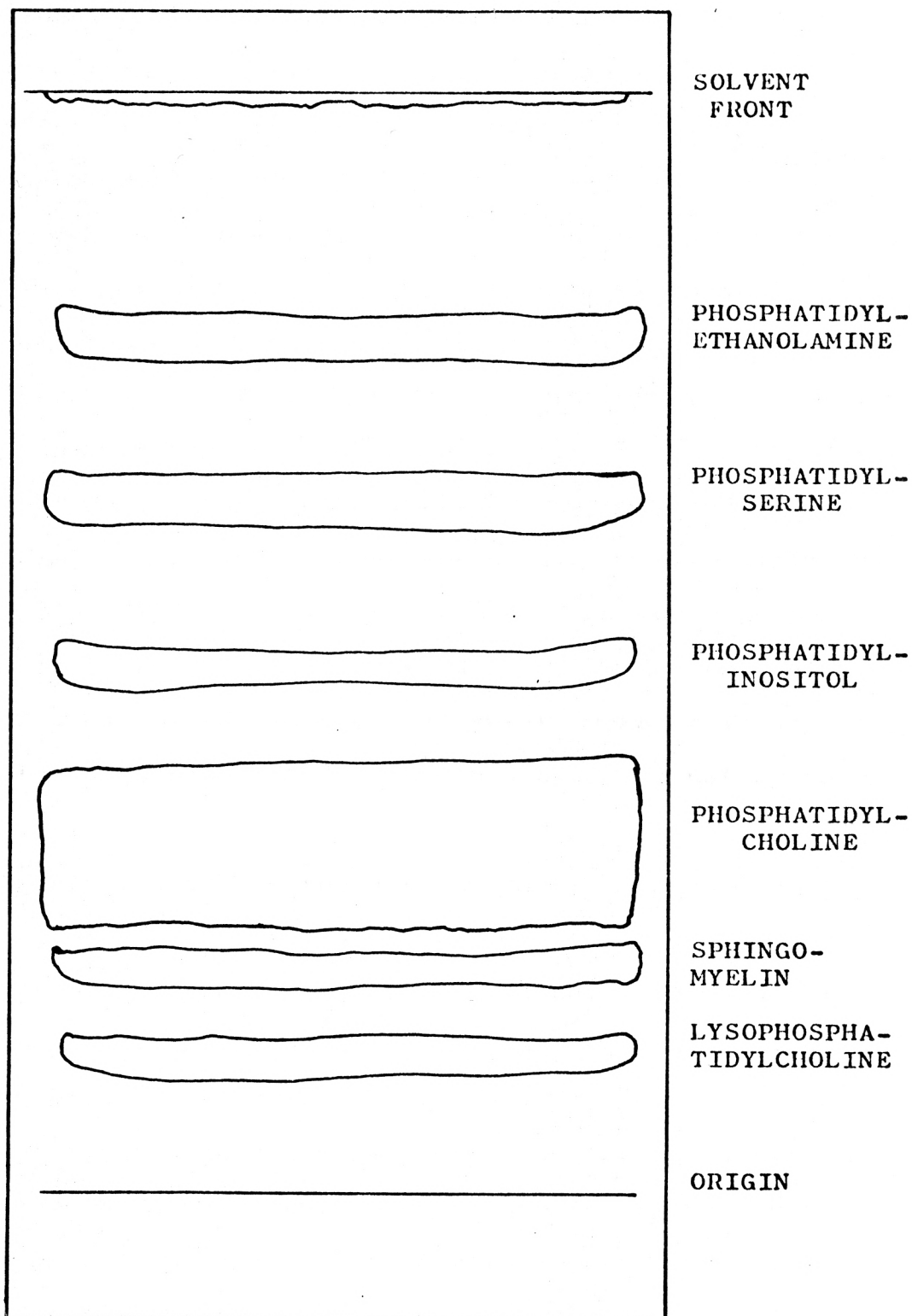


Figure 2. Thin layer chromatograph of the phospholipids of rat liver

tidylcholine and .18 for sphingomyelin. A drawing of a representative thin layer separation of the column eluate is seen in Figure 2. The phosphatidylcholine bands were quite broad and some separation into subclasses was occurring, as was also reported by Lyman et al. (9). It was therefore important to scrape the bands as quantitatively as possible and this was checked by exposing the scraped plates to iodine vapors. Failure to obtain a representative sample could cause an apparent change in the composition.

The chromatography of the diglycerides obtained after phospholipase C hydrolysis to separate 1,2 from 1,3 diglycerides was eliminated because, after only a 12 hour reaction, the production of the 1,3 diglycerides was minor and the GLC of the TMS derivatives did not give split peaks or in any way indicate the presence of 1,3 diglycerides contaminating the 1,2 diglycerides.

The weakest point in the attainment of the results was the reliability of the gas chromatography. The characteristics of the column material varied whether obtained from the same or a different supplier. The original plan to use 3% OV-1 on Gas Chromosorb Q 100/120 mesh was abandoned when this failed to give baseline resolution of the two subfractions of the 36 carbon species as had previously been obtained (59). In a personal communication with J. F. O'Brien it was suggested to try the 3% Apolar-10C packing material. The 10% Apolar-10C was tried on the assumption that it might

give better resolution, and it did. This material gave baseline resolution of several molecular species of both 34 and 36 carbons differing in the number of double bonds, and it gave better resolution and faster chromatographic times than the recently reported system of Myher and Kuksis (56) using 3% Apolar-5CP, although the general pattern of resolution was similar.

The retention times of the standards indicate that the retention is indeed a function of not only the number but also the distribution of the double bonds in the molecule. This can be seen most vividly by referring to Figure 3. The retention information is also shown in Table 1. It is evident that the standards 18:0, 18:1; 18:1, 18:1; and 18:0, 18:2 are all resolved from one another. All these molecular species have a total of 36 carbons and differ from each other only in the number or distribution of the double bonds. The resolution of 18:0, 18:1 from 18:1, 18:1 and 18:0, 18:2 is quite remarkable. Even more striking however, is the resolution of 18:1, 18:1 from 18:0, 18:2. These species not only have the same total carbon number, but also have the same number of double bonds. They differ only in the distribution of the double bonds within the molecule, the former having one double bond in each chain and the latter having both double bonds in the same chain. It will also be noted from this table that two double bonds, one in each chain, appears to have about the same effect on retention time as the addition

Table 1. Retention of commercial diglyceride standards

CARBON SPECIES*	FATTY ACIDS PRESENT	RETENTION ^o (cm)	RETENTION ^Δ (cm)
28:0 ⁺	14:0,14:0	4.4	3.5
32:0	16:0,16:0	7.6	5.8
32:2	16:1,16:1	10.1	7.7
34:0	17:0,17:0	10.1	7.3
36:0	18:0,18:0	13.6	9.7
36:1	18:0,18:1	14.9	10.7
36:2	18:1,18:1	16.7	12.0
38:0	19:0,19:0	18.2	12.9
36:2	18:0,18:2	20.3	14.7
36:4	18:2,18:2	23.0	17.0
36:6	18:3,18:3	33.2	23.7

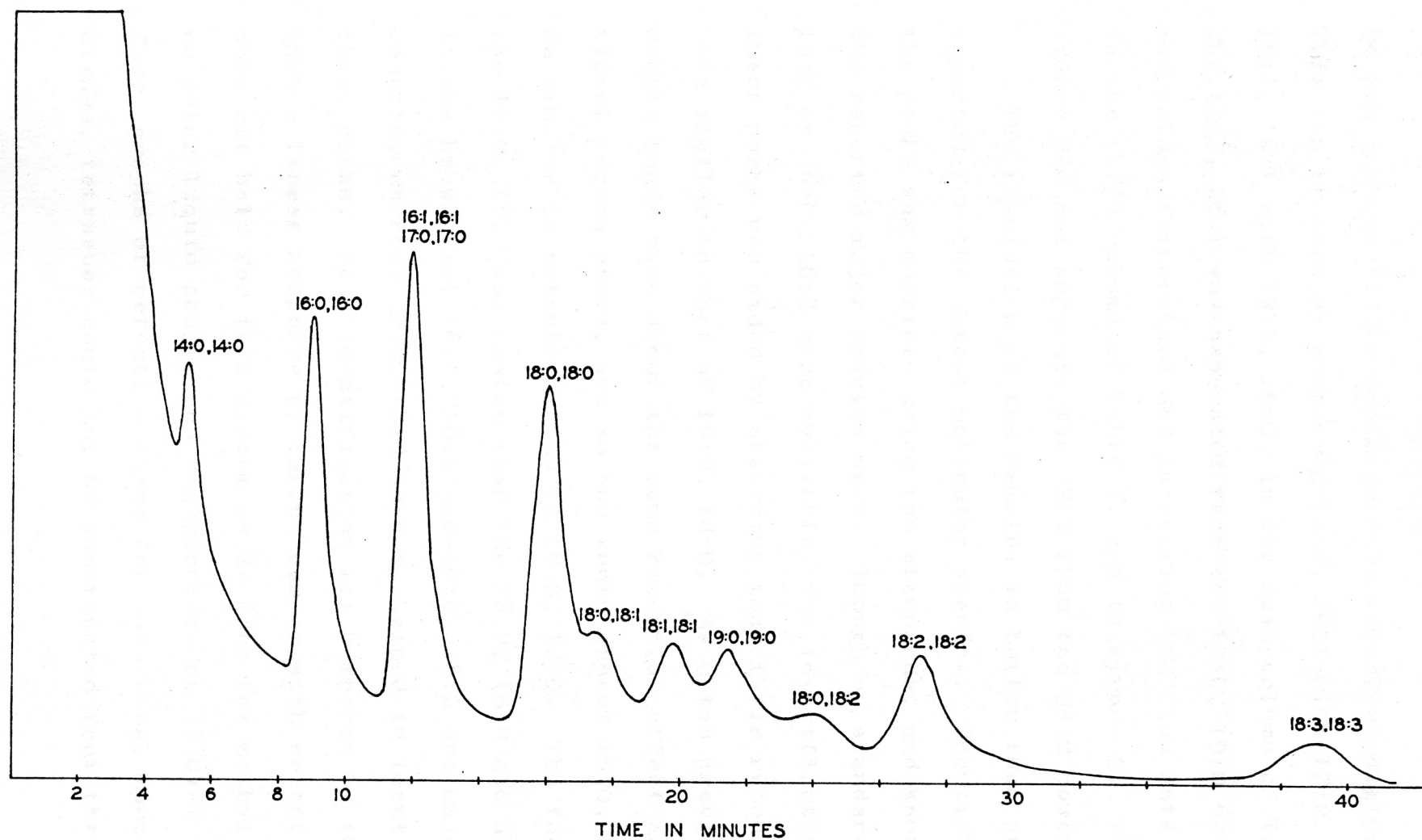
*Only carbons of the two fatty acids are included

+The number before the colon indicates the number of carbons present in the two fatty acids, and the number after refers to the number of double bonds.

^oColumn temperature at 257° C, flow rate of 75 ml/min.; pooled standards chromatographed

^ΔColumn temperature at 265° C, flow rate of 40 ml/min.; individual standards chromatographed

Figure 3. Gas chromatograph of commercial diglyceride standards.



of two carbons to the corresponding saturated diglyceride. This can be seen by comparing 16:1, 16:1 with 17:0, 17:0, and 18:1, 18:1 with 19:0, 19:0, in the last column of Table 1. The 18:1, 18:1 was separated from the 19:0, 19:0 by lowering the column temperature and increasing the flow rate as shown in the third column of Table 1, and in Figure 3. This procedure did not separate the 32:2 from the 34:0 however.

The resolution of the samples is better than previously reported for the intact molecular species. Identification of the peaks was possible using the standards, and knowing what the reported major species were. Though no standards of 16:0, 18:1 or 16:0, 18:2 were available, the identification of these peaks was aided by observing that their retention times were similar to that of 18:0, 18:0. As noted previously, two double bonds have about the same retention effect as two additional carbon atoms, and so one would expect 16:0, 18:2 to be similar in retention time to 18:0, 18:0. The fact that the 16:0, 18:2 was faster than the 18:0, 18:0 and also that it was known that 16:0, 18:1 and 16:0, 18:2 are major components, whereas 18:0, 18:0 is not, helped in identifying these peaks. Peak identification was hampered by the fact that a linear response of carbon number with retention time does not hold for this system as it does for methyl esters on polar liquid phases and diglycerides on 3% OV-1 (59). Thus, graphs of retention times for saturates, monoenes, dienes, tetraenes could not be constructed from the standards.

The identity of some of the minor components could not be determined with certainty because standards were not available.

It appears that this system has two major drawbacks. First, the hexaene species are retained quite tightly. Again, noting from the retention times of the standards, as the distribution of the double bonds shifts to a preponderance in one chain, the retention time increases. Thus, as 18:0, 18:2 is retained longer than 18:1, 18:1, it would appear that a 16:0, 22:6 should be retained longer than an 18:3, 18:3 as it has two additional carbons and the six double bonds occur in one chain. Thus, even though the 18:3, 18:3 did eventually come through, no indication of a 16:0, 22:6 peak appeared on any of the samples. Myher and Kuksis (56) did report that their system did elute the 16:0, 22:6, but the 18:0, 22:6 was lost. As the packing material used in this experiment is roughly twice as polar and exhibits a stronger retention of double bonds, one might expect that the 16:0, 22:6 would not be eluted by the system used in this research. The other drawback to this system is the short lifetime of the column. After a three day conditioning period a column would give good chromatograms for only four to six days. Peak shapes then deteriorated and resolution rapidly became unacceptable.

As the samples were stored over a period of months, a check for autoxidation (62) was made on a number of random samples. No evidence of autoxidation appeared. As a further

check, the procedure was carried through from liver excision to gas chromatography within a 24-hour period, and the results obtained were similar to the control results, again indicating no significant effect of storage.

A comparison of the control values with others reported in the literature for individual molecular species is limited, since quantitation of such species has been up to now only limitedly achieved by reverse phase partition chromatography. Two recent reports can be cited, however. Dyatlovitskaya et al. (63) and Myher and Kuksis (56) reported values for all the major and some minor molecular species of phosphatidylcholines from normal rat liver. Discrepancies between their data and the data shown in Table 2 are apparent and so the correctness of the percentages of each molecular species in Table 2 is not discernable. The major differences occur in the species 16:0, 18:2 and 18:0, 18:2. The comparison of the control values obtained in this research to those of others will show a difference because no hexaene species were determined. The percentages of the other species will be artificially increased by the omission of the hexaene species. This may account for the differences seen in the 16:0, 18:2 and 18:0, 18:2 species.

By grouping the percentages according to monoene, diene, and tetraene, more comparisons can be made. Comparisons of the hexaene species cannot be made. Widespread agreement of the percent composition of the monoene subfraction is found.

Table 2. Percentages of the molecular species of phosphatidylcholines after the appropriate drug treatment.

MOLE- CULAR SPECIES	CONTROL	ETHIO- NINE	PHENO- BARBITAL	ETHIO- NINE plus PHENO- BARBITAL
16:0,16:0	2.2 \pm .7	2.1 \pm .3	.7 \pm .6	1.1 \pm .4
16:0,16:1	.6 \pm .2	.9 \pm .2	1.1 \pm .5	1.4 \pm 1.0
---	.7 \pm .4	.6 \pm .3	.8 \pm .6	1.2 \pm .7
16:0,18:1	9.4 \pm 1.0	9.2 \pm .5	7.2 \pm 2.0	8.6 \pm 1.6
16:0,18:2	26.3 \pm 1.1	39.2 \pm 2.4	27.3 \pm 2.3	32.5 \pm 0
18:0,18:1	2.0 \pm .5	3.2 \pm .5	2.4 \pm .1	3.5 \pm 2.4
18:0,18:2	12.4 \pm 1.8	19.8 \pm 1.4	15.1 \pm 1.8	21.0 \pm 1.2
16:0,20:4	22.4 \pm 3.7	15.0 \pm 1.2	18.4 \pm 3.1	16.2 \pm 1.8
---	1.6 \pm .9	1.8 \pm .5	4.4 \pm .4	2.1 \pm .2
18:0,20:4	20.5 \pm 1.6	9.3 \pm .9	16.6 \pm 1.1	12.2 \pm 3.7
---	2.9 \pm 1.3	n.p.	2.6 \pm .9	n.p.

---Indicates that a determination of the identity of the peak was not possible

n.p.— not present

Several groups (8,12,14,63) all report 11% monoenes, with Lyman et al. (10,20) reporting 8.5 and 9.8%. Combining the 16:0, 18:1 and 18:0, 18:1 percentages of Table 2 gives 11.4% for the monoenes. Reported values for dienes are 28% (8), 25% (12), 23.5% (14), 31% (63), 30.7% (10), and 22.5% (20). The data of 16:0, 18:2 and 18:0, 18:2 from Table 2 gives a somewhat higher value of 38.7%. Values for the tetraene species show even wider variations with values from 35% (8) to 58.2% (10) being reported. The data for 16:0, 20:4 and 18:0, 20:4 give a 42.9% value that falls in the middle of the reported values. These results seem to indicate that the values for the monoenes and tetraenes are within the proper limits, while the dienes appear to have somewhat higher values than most reports. Hexaene values have been reported from 5% (8) to 16% (20).

By examining the data in Table 2 and comparing Figures 4 and 5 it is seen that ethionine treatment increases the percentages of 16:0, 18:2 and 18:0, 18:2 while decreasing those of 16:0, 20:4 and 18:0, 20:4. This is in good agreement with the results reported by Lyman et al. (30,64). They found that ethionine reduced the percentage of arachidonic below control levels in both males and females, while elevating the percentage of linoleic. In males oleic acid was also increased. Ariyoshi et al. (47) also reported that ethionine decreased arachidonic and increased linoleic acid in female phosphatidylcholines. Ethionine as an inhibitor

of the methylation pathway should decrease the amount of phosphatidylcholine synthesized by this pathway. Ethionine appears to be acting at other sites also. Ulsamer and Glenn (28) had earlier shown that ethionine decreases the incorporation of ^{32}P orthophosphate into phosphatidylethanolamine and increases the incorporation into phosphatidylcholine. They also demonstrated that normal rats rapidly incorporate the label into phosphatidylethanolamine, but it is only slowly incorporated into phosphatidylcholine. They suggested that ethionine decreases the synthesis of phosphatidylethanolamine and stimulates the synthesis of phosphatidylcholine. Sato and Hasegawa (29) speculate that the decreased labelling into phosphatidylethanolamine is a result of decreased synthesis of phosphatidylethanolamine from ethanolamine. This is brought about by feedback inhibition of phosphatidylethanolamine due to its decreased conversion to phosphatidylcholine. They also suggest that the synthesis of phosphatidylcholine via the Kennedy pathway is stimulated as a compensatory mechanism. Furthermore, Lyman et al. (30) demonstrated that ethionine appeared to decrease the desaturation reaction in the conversion of linoleic to arachidonic acid. All these effects would contribute to the increase in linoleic acid and the decrease in arachidonic acid brought about by ethionine.

The change in percentage of the fatty acids caused by ethionine would lead one to suggest that the species con-

taining arachidonic acid are formed mainly via the methylation pathway. The literature also suggests that the hexaene fraction may be more dependent upon the methylation pathway than the tetraene fraction in the synthesis of phosphatidylcholine. Lyman et al. (9) reported that the fatty acid composition of the two fastest moving phosphatidylcholine fractions on thin layer plates resembled the distributions in phosphatidylethanolamine, and they contained more arachidonic and less linoleic than the slower moving phosphatidylcholine fraction. Administration of ^{14}C -methionine to females showed that the phosphatidylcholine subfractions containing arachidonic acid preferentially incorporated the label. Later, Lyman et al. (10) reported that estradiol given to castrated male rats injected with ^{14}C -methionine incorporated more label than controls, and the largest part of the label appeared in the tetraene subfraction. This subfraction contained the hexaene species also. Analysis of the fatty acids in phosphatidylcholine showed that estradiol increased the percentage of the polyenoic fatty acids (22:5, 22:6), but did not alter the percentage of arachidonic acid. Estradiol also decreased the percentage of 16:0 and 18:2. The effects of estradiol and methionine on the methylation pathway are antagonistic and the percentages of certain fatty acids reflect these effects. Estradiol also appears to affect the synthesis of phosphatidylethanolamine in addition to its conversion to phosphatidylcholine. Bjørnstad and Bremer (4) had previously re-

ported that females incorporate more ethanolamine into their phosphatidylethanolamine and phosphatidylcholine than males, presumably due to the effect of estradiol. In castrated males, estradiol decreased the percentage of linoleic and increased the 22:5 and 22:6 acids in phosphatidylethanolamine (10). Other workers (11,12,14) also demonstrated by incorporation studies of ^{14}C -ethanolamine and ^{14}C -methionine that the hexaene fraction of phosphatidylcholine appeared to be preferentially labelled. It thus appears that the hexaene subfraction of phosphatidylethanolamine is the major methylation substrate, and if methionine is decreasing the synthesis of phosphatidylcholine by this pathway, a decrease in this fraction should be seen. Unfortunately, the system used in this research did not elute the hexaene fraction and so no conclusions as to its fate can be made.

As Young et al. (45) had reported that phenobarbital increased the specific activity of SAME:PE methyltransferase, it was speculated that the diglycerides produced would reflect the enhancement of this pathway and an increase in species containing arachidonic and docosahexenoic acids and a decrease in diglycerides with linoleic acid might be the result. However, the data in Table 2 do not support this speculation. The data demonstrate that phenobarbital decreases 16:0, 20:4 and 18:0, 20:4; increases 18:0, 18:2; and does not alter 16:0, 18:1 and 16:0, 18:2. This data is in agreement with that of Davison and Wills (48) who reported an increase in

linoleic and a decrease in arachidonic and docosahexenoic acids. Again, the effect of phenobarbital on the 16:0, 22:6 and 18:0, 22:6 species is open to question as these were not eluted from the column. From the data of Davison and Wills, one might predict that the docosahexenoic species would be decreased in relation to controls. Ariyoshi et al. (47) did not report any change in the fatty acids after phenobarbital treatment. A sex difference may be the cause as they used females, and males were used in this study and the one by Davison and Wills.

The data in Table 2 do not support the observation that the methylation pathway is increased by phenobarbital as reported by Young et al. (45). Davison and Wills (46) were able to report that phenobarbital did cause an increase in the specific activity of SAME:PE methyltransferase after 12 hours, but the activity returned to normal after two days of treatment and fell below normal after three days of treatment. Various other workers (41,42,43,44), though not measuring the methylation pathway directly, have concluded that phenobarbital decreased the conversion of phosphatidylethanolamine to phosphatidylcholine. The data in Table 2 is consistent with a decrease in the methylation pathway brought about by phenobarbital. However, comparison of the percentages of the molecular species between ethionine and phenobarbital demonstrate that they are not acting in the same direction and are not of the same magnitude in many cases. The effect on the molecular species produced by both drugs bears this out. Compari-

Figure 4. Gas chromatograph of the 1,2 diglyceride TMS derivatives of the phosphatidylcholines from the rat liver of a control animal.

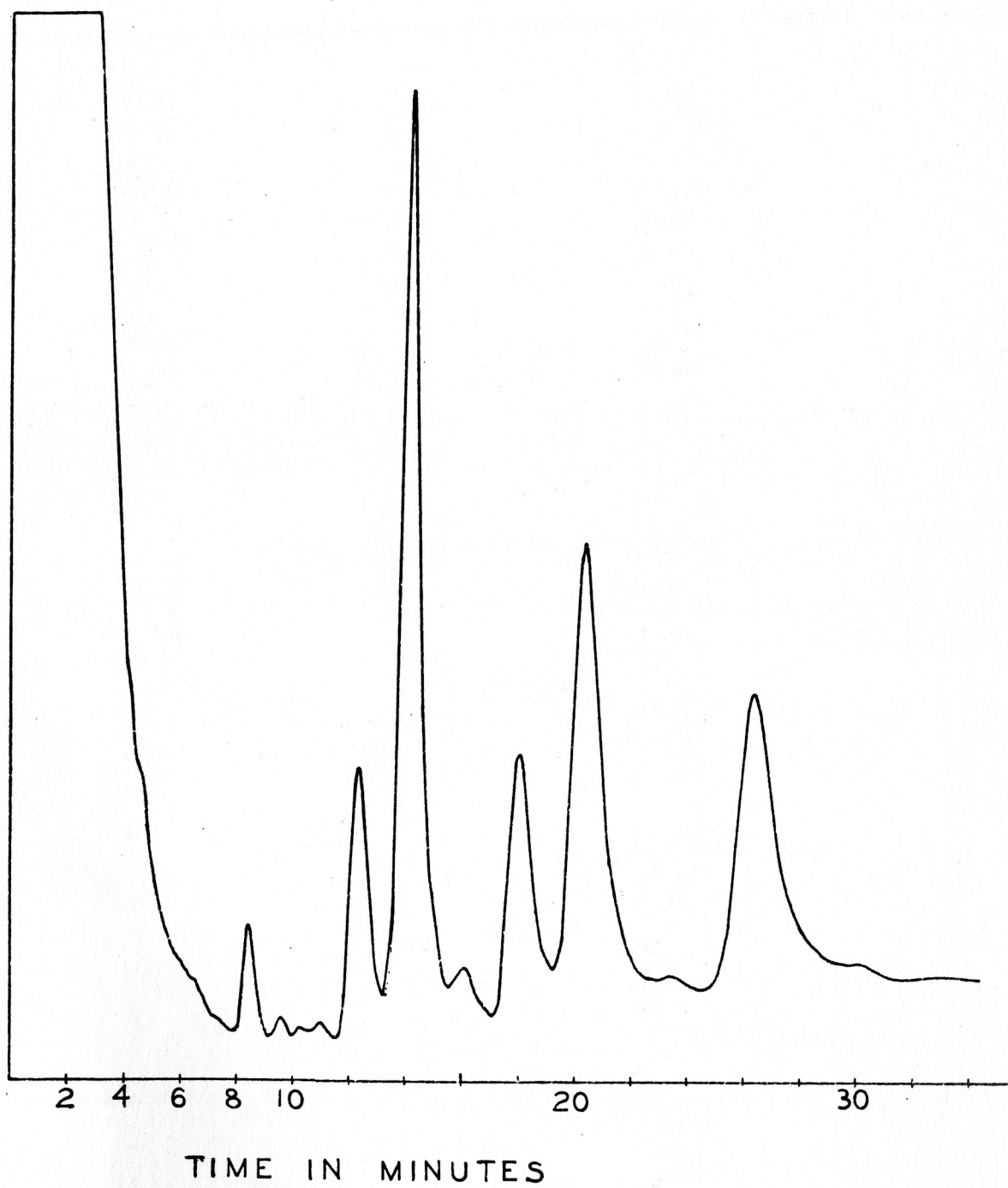


Figure 5. Gas chromatograph of the 1,2 diglyceride TMS derivatives of the phosphatidylcholines from the rat liver of an ethionine treated animal.

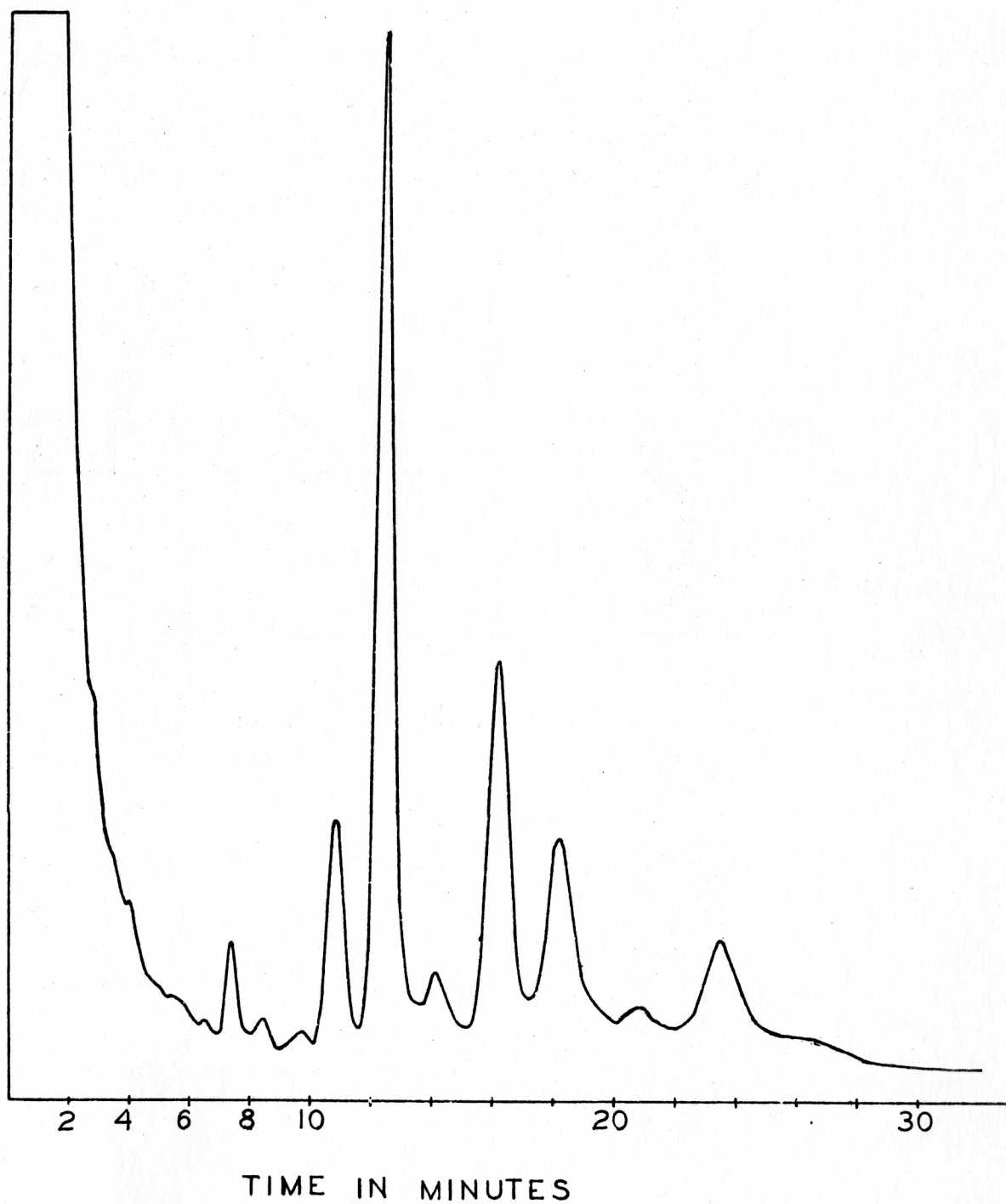


Figure 6. Gas chromatograph of the 1,2 diglyceride TMS derivatives of the phosphatidylcholines from the rat liver of a phenobarbital treated animal.

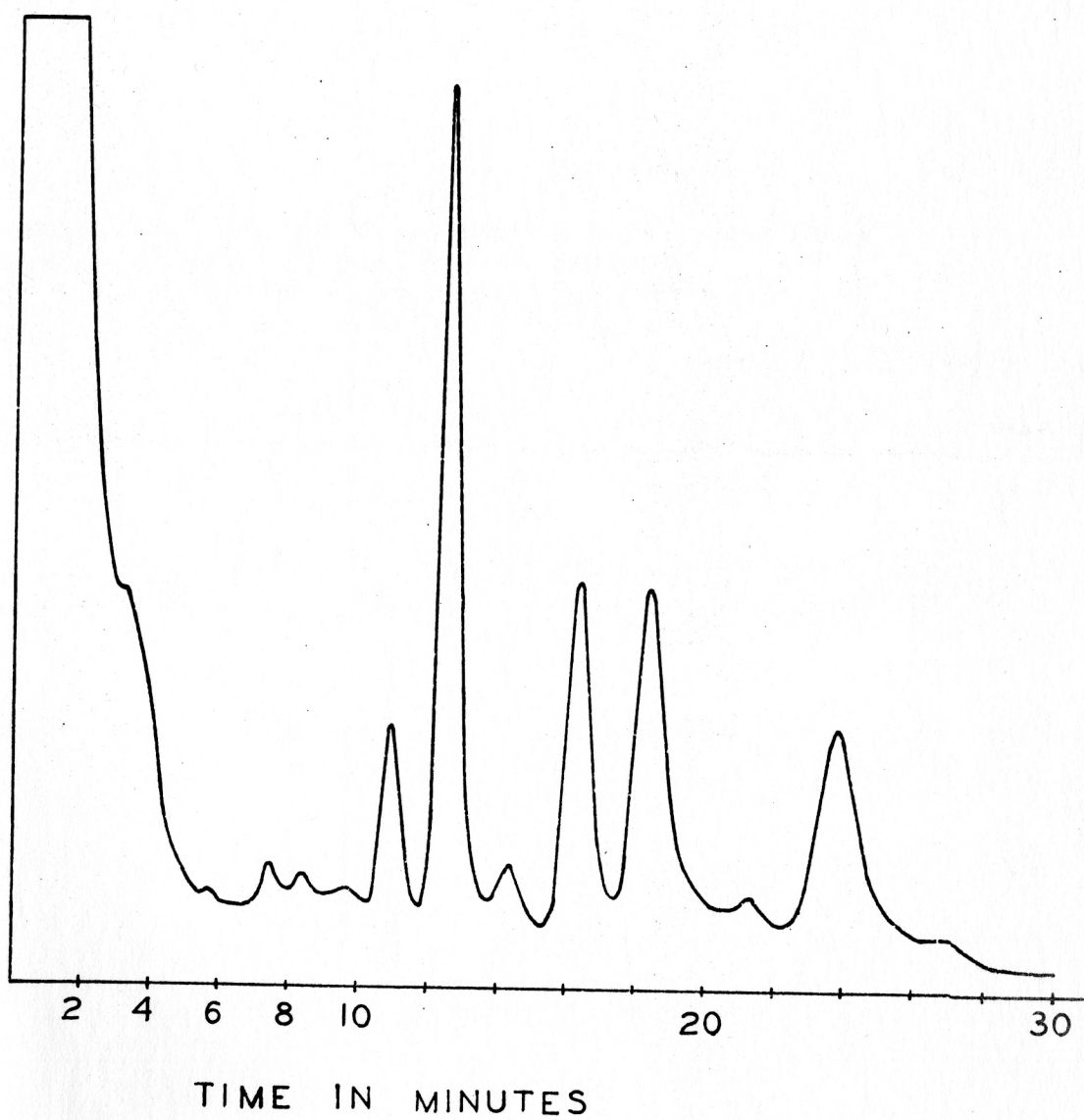
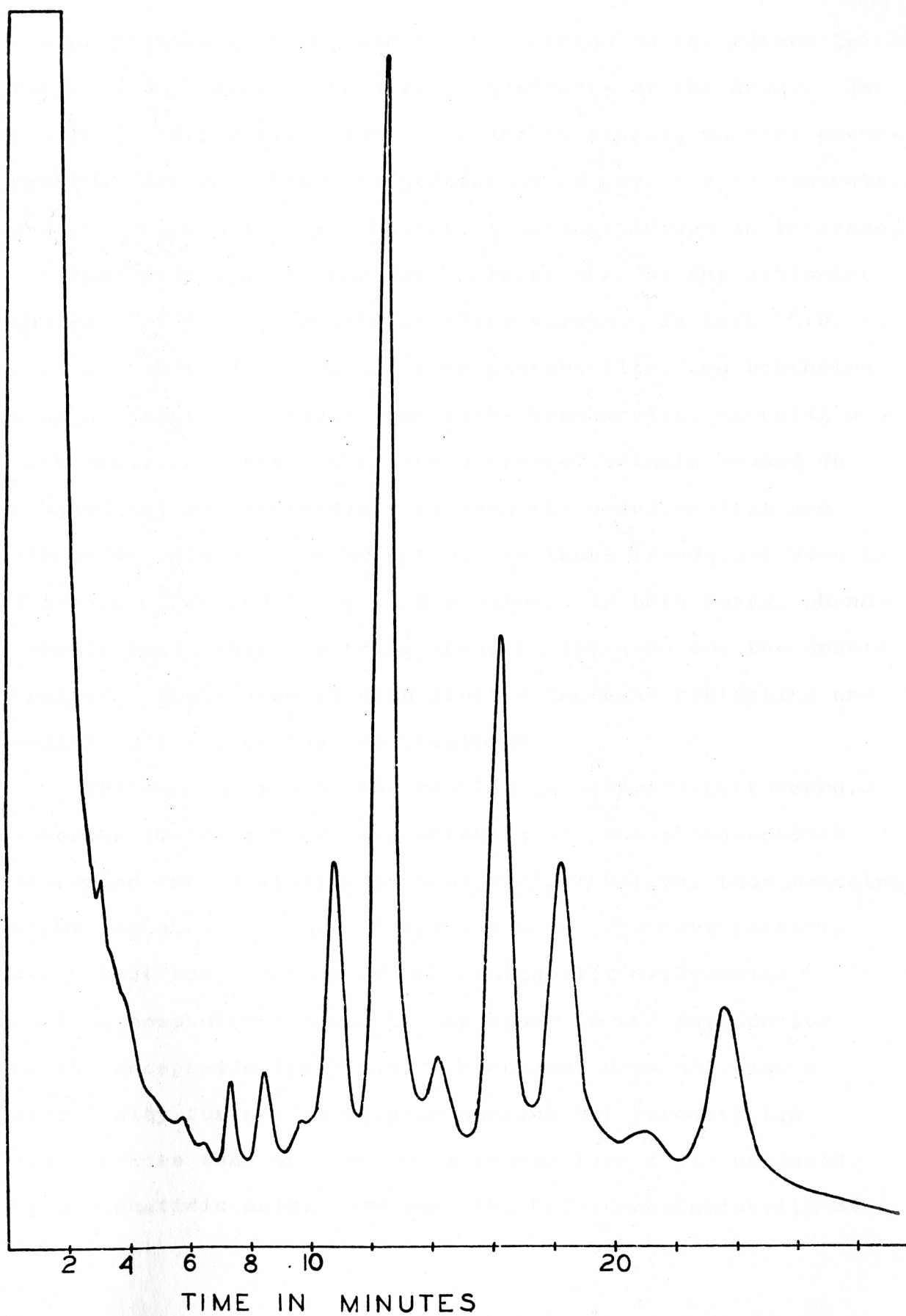


Figure 7. Gas chromatograph of the 1,2 diglyceride TMS derivatives of the phosphatidylcholines from the rat liver of an ethionine and phenobarbital treated animal.



son of Figures 4, 5, 6, and 7, in addition to the values in Table 2, demonstrate the differing effects of the drugs. In the 16:0, 18:2 diglyceride ethionine increased, whereas phenobarbital did not alter the percentage as compared to controls. The phenobarbital plus ethionine treatment showed an increase, but the value was intermediate between that of the ethionine and that of the phenobarbital alone values. In both 16:0, 20:4 and 18:0, 20:4 species both phenobarbital and ethionine brought about a decrease, but with phenobarbital exerting a much smaller effect. The double treated animals showed decreased values intermediate between the phenobarbital and ethionine values. Two exceptions to these trends are seen in the 18:0, 18:1 and 18:0, 18:2 species. In both cases, phenobarbital and ethionine bring about an increase and the double treated animals show an even greater increase reflecting an additive effect of the two treatments.

The explanation of the results is not straightforward. Numerous investigators have established that phenobarbital decreased the catabolism of phosphatidylcholine, thus bringing about its accumulation. Kanoh and Ohno (25) have recently shown that the reaction of CDP-choline:1,2 diglyceride cholinephosphotransferase is important in the degradation of the phosphatidylcholines. The enzyme does not show a specificity for any particular species but produces 1,2 diglycerides that are more unsaturated than those produced by phosphatidic acid. However, the CDP-ethanolamine:1,2 di-

glyceride ethanolaminephosphotransferase is not readily reversible (4), and it does exhibit a specificity for the hexaene species (25). A decrease in the activity of this enzyme would decrease the use of the hexaene species for the synthesis of phosphatidylethanolamine, and would possibly decrease the degradation of this species by the CDP-choline pathway due to feedback inhibition. The combination of these pathways may account for the observation by Arvidson (12) that under normal conditions the hexaene phosphatidylcholines lost the ^{14}C -methyl label most rapidly. Phospholipase B is known to hydrolyze the unsaturated fatty acids faster than the saturated ones from either position 1 or 2. It also deacylates phosphatidylethanolamine faster than phosphatidylcholine (52). Decreases in the activities of these enzymes should cause an increase in the species with more unsaturated fatty acids. The combination of the decreased methylation pathway which would decrease the tetraene and hexaene species of phosphatidylcholine, and the decreased catabolism of these same species which would lead to their increase appears to explain the results produced by phenobarbital.

The intermediate values of the phenobarbital plus ethionine treatment can also be explained by the same hypothesis. Even though both ethionine and phenobarbital have decreased the methylation pathway, the added effect of phenobarbital on the catabolic enzymes would tend to raise the decreased level of the polyenoic species as is seen from the data.

The additive effects of phenobarbital and ethionine on the increases of 18:0, 18:2 and 18:0, 18:1 can also be explained by the decreased catabolic enzymes toward all subspecies. The ethionine increases the formation of phosphatidylcholine by the CDP-choline pathway, and though phenobarbital does not appear to alter the synthesis of phosphatidylcholine by this pathway, the increase seen is due to the decreased effect of the catabolic enzymes. The double treated animals have both an increased synthesis and a decreased catabolism to give the additive effect. The pattern of the 16:0, 18:2 is harder to explain. The increase by ethionine is due to the increased synthesis, while phenobarbital does not alter the percentage, possibly because the catabolic enzymes are still active toward this species. This would explain again the intermediate value of the double treated animals.

Due to the inability of this system to elute the hexaene fractions, the percentage of the other fractions may be somewhat high. The small percentage of that fraction will not alter the other percentages excessively, however. The larger the initial amount of a fraction, the greater the increase in percentage will occur due to the deletion of the hexaene fraction. As the 16:0, 18:2 peak was the largest and the 18:0, 18:2 peak was the fourth largest, this may explain why the comparison of diene values showed the results from this research to be higher than other reported values. The same would hold for the tetraene fraction.

If the hypothesis suggested to account for the changes in diglyceride species brought about by the drug treatments is true, one would predict that the hexaene species would be decreased in all the drug treatments.

Development of systems that will elute the hexaene diglycerides is needed before adequate conclusions about the effects of phenobarbital can be made.

SUMMARY

The diglyceride species of phosphatidylcholines from male rat liver after treatment of the animal with ethionine, phenobarbital or both drugs were determined using gas chromatography on 10% Apolar 10C. Ethionine reduced the percentages of the 16:0, 20:4 and 18:0, 20:4 diglycerides, while increasing those of the 16:0, 18:2 and 18:0, 18:2. Phenobarbital did not alter the 16:0, 18:2, reduced the 16:0, 20:4 and 18:0, 20:4, and increased the 18:0, 18:2 diglycerides, though not as drastically as ethionine. Treatment with both drugs produced intermediate values in all cases except for the 18:0, 18:1 and 18:0, 18:2 diglycerides, where additive effects were seen. It was concluded that phenobarbital decreases the methylation pathway and the activities of the catabolic enzymes to bring about the alterations in diglyceride species. The intermediate and additive values for the ethionine plus phenobarbital treated animals can be explained by the counterbalance produced by the large decrease in the methylation pathway by ethionine and the decreased activity of the catabolic enzymes by phenobarbital.

The diglyceride species were prepared by phospholipase C hydrolysis of the purified phosphatidylcholines. TMS derivatives were made and used for the gas chromatographic procedure. The system used gave good baseline resolution of

all the major diglyceride species, except for the hexaene species which were not eluted.

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STUDIES OF THE EFFECTS OF ETHIONINE AND PHENOBARBITAL
ON THE PHOSPHATIDYLCHOLINES OF RAT LIVER

by

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Male rats were treated by injection with ethionine, phenobarbital, or with both drugs. The livers were removed, and the lipids extracted according to Folch et al. (57). The phosphatidylcholines were isolated by column and thin layer chromatography, and the diglycerides prepared by phospholipase C hydrolysis. Trimethylsilyl derivatives were made of these diglycerides and separated by gas chromatography. An 8 foot by 1/8 inch glass column packed with 10% Apolar 10C on Chromosorb Q 100/120 mesh, and operated isothermally at 265° C was used. Baseline resolution of all the major diglyceride species was achieved, except for the hexaene fraction which was not eluted. Resolution that distinguished not only between the number of carbon atoms and double bonds within the diglyceride, but also in the distribution of the double bonds within the molecule was attained.

Ethionine was found to decrease the percentages of the 16:0, 20:4 and 18:0, 20:4 diglycerides, and increase those of 16:0, 18:2 and 18:0, 18:2 largely by decreasing the methylation of phosphatidylethanolamine to produce phosphatidylcholine. Phenobarbital did not alter 16:0, 18:1 or 16:0, 18:2, decreased 16:0, 20:4 and 18:0, 20:4, and increased 18:0, 18:2. The changes seen were not as large as those brought about by ethionine. It is suggested

that phenobarbital alters the molecular species by both decreasing the methylation pathway and by decreasing the activities of some of the catabolic enzymes. Treatment with both ethionine and phenobarbital produced intermediate values in the 16:0, 18:2; 16:0, 20:4; and 18:0, 20:4 diglycerides. Additive effects were seen in the 18:0, 18:1 and 18:0, 18:2 diglycerides. These effects are a result of the counter balance between the decreased methylation pathway brought about by both drugs, and the decrease in the activities of the catabolic enzymes brought about by phenobarbital.